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PROPHYLACTIC ADMINISTRATION OF PONAZURIL REDUCES CLINICAL SIGNS AND DELAYS SEROCONVERSION IN HORSES CHALLENGED WITH *SARCOCYSTIS NEURONA*

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ABSTRACT: The ability of ponazuril to prevent or limit clinical signs of equine protozoal myeloencephalitis (EPM) after infection with *Sarcocystis neurona* was evaluated. Eighteen horses were assigned to 1 of 3 groups: no treatment, 2.5 mg/kg ponazuril, or 5.0 mg/kg ponazuril. Horses were administered ponazuril, once per day, beginning 7 days before infection (study day 0) and continuing for 28 days postinfection. On day 0, horses were stressed by transport and challenged with 1 million *S. neurona* sporocysts per horse. Sequential neurologic examinations were performed, and serum and cerebrospinal fluid were collected and assayed for antibodies to *S. neurona*. All horses in the control group developed neurologic signs, whereas only 71 and 40% of horses in the 2.5 and 5.0 mg/kg ponazuril groups, respectively, developed neurologic abnormalities. This was significant at $P = 0.034$ by using Fisher exact test. In addition, seroconversion was decreased in the 5.0 mg/kg group compared with the control horses (100 vs. 40%; $P = 0.028$). Horses with neurologic signs were killed, and a post-mortem examination was performed. Mild-to-moderate, multifocal signs of neuroinflammation were observed. These results confirm that treatment with ponazuril at 5.0 mg/kg minimizes, but does not eliminate, infection and clinical signs of EPM in horses.

Equine protozoal myeloencephalitis (EPM) is a commonly diagnosed condition of the central nervous system (CNS) of horses, which results in clinical signs of weakness, gait deficits, and less commonly blindness or seizures (Furr et al., 2002). The condition is most commonly caused by infection with the protozoan *Sarcocystis neurona* (Dubey, Lindsay et al., 2001). Seropositivity to *S. neurona* is high in many areas of the United States, but it varies widely (Blythe, Granstrom et al., 1997; Blythe, Granstrom, and Stamper, 1997; Bentz et al., 2003; Rossano et al., 2003).

Clinical illness resulting from *S. neurona* infection is comparatively rare, however, but when present it results in significant financial losses associated with lost training time, diagnosis, and treatment. For these reasons, there is a strong interest in methods to prevent the development of EPM.

Methods for prevention of EPM are currently ill-defined. Vermin control has been advocated, along with ensuring the security of grain supplies, such that it does not become contaminated with infected feces (Johnson, 1998). A vaccine has been developed and marketed; however, the efficacy of vaccination has been questioned and has not been proven, to date, by prospective evaluation. Treatment with pyrantel tartrate has been attempted, but it has been found to have no effect upon infection (seropositivity) in immunocompetent horses (Rossano et al., 2005). Therefore, at present there is no proven method of prevention.

Ponazuril has been found to be effective for the treatment of clinical EPM (Furr, Kennedy et al., 2001), and it has been shown to kill *S. neurona* in vitro (Lindsay et al., 2000). Prophylactic treatment with ponazuril should kill *S. neurona* organisms in the bowel or during a peripheral tissue growth phase, before infecting the CNS and inducing clinical illness. The present study was performed to determine whether prophylactic administration of ponazuril results in a reduction in the incidence or severity of EPM in horses after challenge with *S. neurona*.

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MATERIALS AND METHODS

Horses

The study was evaluated and approved by the Virginia Tech Animal Care and Use Committee and was performed in compliance with all applicable state and federal regulations. Twenty horses, 4 to 7 mo old, were acquired from a commercial source in Manitoba, Canada. To select horses, candidate animals were examined for general health and screened by serum Western blot analysis (WB) for antibodies to *S. neurona*. A neurologic examination was performed by 2 of the authors (M.F. and W.S.) on those horses that were serum WB negative. From this pool, 20 horses (10 males and 10 females), which were considered by both investigators to be neurologically normal, were selected. Selected horses were assigned to 1 of 3 treatment groups: group 1, control (ponazuril 0 mg/kg); group 2, low dose (2.5 mg/kg ponazuril, oral, daily); or group 3, high dose (5.0 mg/kg ponazuril, oral, daily). Assignment to treatment group was by random draw method, with 6 horses assigned to each treatment group. Two additional horses were selected and randomized into a group to serve as replacements should a horse have an injury or illness associated with shipping, which made them unsuitable for the study. After selection, horses were kept at the farm in Manitoba until shipment to the primary study site in the United States. After shipping, 2 horses were considered unacceptable and were removed from the study. Therefore, after selection and transport, horses that were entered in the study had an average body weight of 226.8 kg ($\pm$25), and there were 9 males and 9 females. Fourteen of the horses were draft or draft crosses, while 4 were Quarter Horse crosses.

While at the primary study site, horses were fed heat-treated, pelleted feed and grass hay, and they were provided water ad libitum. Foals were kept in stalls with solid walls to the floor and bedding consisted of shavings. The foals were allowed access to a dry-lot paddock for exercise (approximately 1 hr) while stalls were being cleaned each day. These measures were considered likely to minimize, but not eliminate, the possibility of natural exposure to *S. neurona*.

Treatment

Horses were treated with ponazuril (Marquis, Bayer Animal Health, Shawnee, Kansas) at the assigned dosage for a period of 1 wk before shipment, then treatment was continued for the duration of the study (28 days after challenge). The calculated dosage of ponazuril paste was weighed in single-dose syringes to ensure accuracy of the dose to be given. Dosing was performed by placing the dose syringe through the interdental space and depositing the dose of paste on the base of the tongue. The horse was observed for 5 min after dosing to ensure that

there was no loss of medication. A vehicle control paste in a volume equal to the dosage volume of the 5.0 mg/kg dose was administered to horses in the control group (0.0 mg/kg ponazuril).The person performing the treatments was not blinded to group assignment but was not involved in making a determination of daily health status or neurologic evaluations.

Sarcocystis neurona inocula

The *S. neurona* sporocysts were obtained from the intestines of laboratory-reared opossums that were fed muscle from laboratory-raised raccoons inoculated with SN 37-R isolate of *S. neurona* (Sofaly et al., 2002; Stanek et al., 2002). Sporocysts were counted in a hemocytometer and diluted to contain 1 million sporocysts in 40 ml of sterile saline. The sporocysts used had been stored for 60 days at 4 C before feeding to horses and their infectivity was verified in γ-interferon knockout (IFN-KO) mice as described previously (Saville et al., 2001). Mice were given 10,000 sporocysts, and there was complete and uniform fatality.

Sarcocystis neurona challenge

The transport stress model was used to induce EPM (Saville et al., 2001). Horses were shipped from Manitoba, Canada, to the Equine Medical Center (EMC) in Leesburg, Virginia, in a standard stock trailer. Horses were allowed rest twice during the trip and were unloaded for feed, hay, and water. Upon arrival at the EMC, the horses were unloaded and placed into stalls with fresh hay and water. A physical examination was performed to ensure no illness or injury resulted from transport. Procedures as described below were performed, after which a nasogastric tube was passed, and each horse was dosed with 1 million *S. neurona* sporocysts.

Collection of samples

Blood and serum: Upon arrival at the EMC on study day 0, and on days 14 and 28, a complete blood cell count (CBC) was performed on jugular venous blood collected using sterile evacuated tubes. The CBC was performed using an automated cell counter (Pentra60, ABC Diagnostics, Irvine, California), and a differential cell count of 100 white blood cells was performed on a Diff-quick-stained blood smear by an experienced hematology technician. In addition, serum was harvested after clot formation at room temperature, and a serum chemistry analysis was performed on study day 0 and 28 by using an automated chemistry analyzer (VetAce, Alfa Wasserman, Inc., West Caldwell, New Jersey). In addition, jugular venous blood was collected on study days 0, 7, 14 21, and 28, and serum was harvested. Aliquots of serum were stored at −70 C until the completion of the study at which time it was analyzed by WB assay for antibodies to *S. neurona*. The WB was performed at a commercial laboratory (Equine Biodiagnostics, Inc., Lexington, Kentucky) in which all personnel performing and interpreting the tests were unaware of treatment group assignment.

Cerebrospinal fluid: To collect cerebrospinal fluid (CSF), horses were anesthetized by premedication with xylazine (Anased, Lloyd Laboratories, Shenandoah, Iowa) (1.1 mg/kg, intravenously [IV]), followed by ketamine (Ketaved, Vedco, St. Joseph, Missouri) (2.2 mg/kg IV), and a CSF sample was collected from the atlanto-occipital space by using routine clinical methods (Mayhew et al., 1977). Cerebrospinal fluid was examined for color and clarity, and was assayed for nucleated and nonnucleated cell counts by using a hemocytometer counting chamber, and a differential count was performed on cells stained with Wrights stain after processing in a cytocentrifuge (CytoPro, Wescor, Logan, Utah). Total protein was evaluated with an automated clinical chemistry analyzer (VetAce, Alfa Wasserman, Inc., West Caldwell, New Jersey).

Neurologic examination: Neurologic examination was performed by 2 investigators (M.F. and H.M.) with experience in the evaluation of neurologic disease of horses, and who were blinded to the treatment group assignment of all horses. After neurologic examination was performed following routine clinical methods, an ataxia severity score (0–5) was assigned according to a standard clinical method (Mayhew et al., 1978). The neurologic examination was performed on study day 0 and then repeated weekly thereafter for the duration of the study. For a horse to be considered to have neurologic disease, it had to be considered abnormal by both blinded investigators, scoring the horse independently.

Post-mortem evaluation: At the completion of the study, animals that were considered to have conclusive neurologic abnormalities (abnormal by both evaluators) were humanely destroyed by intravenous administration of commercial euthanasia solution (Beuthanasia-D, Schering-Plough Animal Health, Union, New Jersey), and a post-mortem examination performed. The brain and spinal cord were removed and placed in 10% buffered neutral formalin for fixation. After fixation, the brain was examined, and sections were taken in a stereotypical manner, encompassing multiple sections of the cerebrum and cerebellum, brainstem, and limbus region as well as a minimum of 6 sections of the spinal cord. In addition, the brain and spinal cord were examined grossly by sectioning every 0.5–1 cm and any grossly observable abnormalities were harvested. Tissues were paraffin-embedded then sectioned 5 μm thick and stained with hematoxylin and eosin before evaluation by light microscopy.

In addition to routine histologic examination, immunohistochemical (IHC) staining of nervous tissue was performed. Paraffin-embedded sections of CNS were reacted with anti-*S. neurona* antibodies as described previously (Dubey and Hamir, 2000). The prosector and pathologist as well as the technician performing and reading the IHC stains were not aware of the animal's treatment group status.

Statistical analysis: Statistical analysis was performed using a desktop computer and commercial statistical analysis software (SAS, SAS Institute, Cary, North Carolina). After data entry and validation, descriptive statistics were performed for all continuous variables. The proportion of horses demonstrating neurologic illness was compared among treatment groups by using Fisher exact test, due to the small group sizes. Fisher exact test also was used to determine whether there was a difference among the proportion of horses that seroconverted in the different treatment groups. Continuous data were analyzed by repeated measures analysis of variance, with posterior pairwise comparisons performed using Tukey's adjustment for multiple comparisons. Distribution of data was evaluated by examining a plot of the residuals, to ensure that the conditions of the analysis of variance were met.

RESULTS

Horses seemed to be clinically normal upon receipt at the EMC. Two horses were lame, and they were eliminated from the study. This resulted in unbalanced group sizes. The lame horses returned to normal within 48 hr with no specific treatment. Complete blood cell counts and serum biochemistry analyses were unremarkable in all animals, with occasional values outside of normal limits, but not representing any consistent pattern of illness. On study day 8, 1 horse of treatment group 3 (5.0 mg/kg ponazuril) was noted to have a severe colic associated with large colon tympany. The horse was treated with nonsteroidal anti-inflammatory drugs (flunixin meglumine, 1.1 mg/kg IV) (Banamine, Schering-Plough), antibiotics (enrofloxacin, Bayer Animal Health), and cecal trocharization. The horse survived the colic, but subsequently developed peritonitis and diarrhea, and a large abscess in the body wall. It was removed from the study due to the severity of illness, and results from this animal were not included in statistical analysis of the data. Other than the horse that had severe colic, all other horses remained systemically healthy, active, and maintained a good appetite during the study, with the obvious exception of the neurologic illness. All horses gained weight during the study period, with an average weight gain of 17.7 kg (±7.7).

Neurologic examinations were performed, and all horses were considered to be neurologically normal (ataxia severity score 0) on study day 0. Mild neurologic gait deficits (score 1/5) were noted on day 7 after inoculation, with 2 horses demonstrating neurologic severity scores of 2. By day 14, 5 of 5 horses (100%) in the control group were found to have neurologic gait deficits of grade 1–2 in severity. Over the entire 28-day examination period, 5 of 7 horses (71%) in the 2.5 mg/kg

group developed neurologic abnormalities, and 2 of 5 (40%) horses in the 5.0 mg/kg dose group were affected. The difference in incidence between the 3 treatment groups was statistically significant ($P = 0.034$) (Fig. 1). Of those horses that developed neurologic signs, the number of days to first observation of clinical abnormalities did not differ between treatment groups. The mean severity score of horses in the 5 mg/kg treatment group was significantly less than that of the control horses on day 14 ($P = 0.027$), whereas the mean severity score in horses receiving 2.5 mg/kg was also less than control horses ($P = 0.05$) (Fig. 2). Interestingly, on day 21, the number of horses in the control group that were demonstrating neurologic abnormalities was reduced and then increased again by day 28. This biphasic response was not noted in horses of the 2 ponazuril-treated groups (Figs. 1, 2). Neurologic severity in most foals progressed over a 1- to 2-wk period and then decreased.

The neurologic severity scores reported from the 2 examiners was remarkably similar, with only 8 of 85 (9.4%) total observations discordant. Neurologic scores for the 2 examiners differed by a value of 1 in 7 out of the 8 discordant observations and differed by a value of 2 in the single remaining case.

Western blot analysis found horses to seroconvert beginning by day 14. Five of 5 horses (100%) in the untreated (control) group seroconverted by the end of the study, whereas 4 of 7 horses (57%) in the 2.5 mg/kg dose group seroconverted by day 28. Two of 5 horses (40%) from the 5.0 mg/kg treatment group seroconverted during the study. The proportion of horses that seroconverted did differ by treatment group ($P = 0.028$) when examined by Fisher exact test (Fig. 3).

Positive CSF WB status was not detected in any horse before day 28. At day 28, 3 of 5 horses in the control group were positive, whereas 2 of 7 in the 2.5 mg/kg group had CSF antibodies and 2 of 5 horses in the 5.0 mg/kg dose group had CSF antibodies to *S. neurona* detectable by WB. These proportions did not differ by treatment group ($P = 0.11$) (Fig. 4).

Red blood cell (RBC) counts in CSF remained normal during the study in all horses. Values for RBC and nucleated cell count are summarized in Table I. High CSF RBC numbers were noted in 3 samples that were collected during the study (>300 cells/μl). These values were considered to be due to blood contamination of the sample and are not a reflection of CNS hemorrhage; these values were considered outliers and were removed from statistical evaluation. All 3 of these samples were WB negative. There was no difference in RBC counts between treatment groups or study day ($P = 0.3$). Nucleated cell counts also remained normal in all horses at all time points, with the exception of 1 horse at 1 examination period (cell count 12 nucleated cells/μl). Statistical evaluation did not detect differences in these values for day ($P = 0.3$) or treatment group ($P = 0.2$). Cerebrospinal fluid total protein values were within normal limits at study day 0 and remained unaltered during the study period in all horses. Individual affected horses did not have an increase in CSF total protein at any point during the study.

Post-mortem examination of affected horses did not reveal any grossly visible abnormalities of the CNS. Microscopic evaluation, however, found all affected horses to have neuroinflammatory lesions of variable severity in several areas of the CNS. Lesions consisted of lymphocytic/plasmacytic perivascular cuffing and gliosis. No organisms were detected on histologic examination, and IHC staining was negative.

DISCUSSION

Results of this study demonstrate that pre- and continuous administration of horses with ponazuril at 5.0 mg/kg reduces development of neurologic signs after challenge with *S. neurona* as well as decreasing the infection and subsequent seroconversion. In this study, the uniform development of neurologic signs in control (nontreated horses) confirms the effectiveness of the induction method and was contrasted with only a 40% attack rate in horses treated with 5.0 mg/kg ponazuril. An intermediate attack was found in horses given 2.5 mg/kg ponazuril.

The development of neurologic abnormalities in the horses of the control group is similar to the clinical signs reported in other studies by using the transport stress model of disease induction (Sofaly et al., 2002). In the study reported by Sofaly et al. (2002), gait deficits were noted beginning at day 15 in horses given 10^6 sporocysts and achieved a severity score of 2–3 (Sofaly et al., 2002), whereas in another study using a second transport and a large inoculum, clinical signs were noted at day 5–7 postinoculation (Saville et al., 2004). Hence, in the horses of the present study, neurologic gait deficits were consistent with those noted in previous studies by using the transport stress model.

Neurologic abnormalities also were noted by day 7 in some horses of the 2 treated groups; however, the severity of signs was less, which was consistently noted during the study, with the exception of 1 study day (day 21). Severity of clinical signs was statistically less on day 14 in the 5.0 mg/kg treatment group compared with the control group. This biphasic response, worsening and then improving briefly before worsening again, has not been reported in previous studies using the transport stress model. A mild biphasic response was noted in a study using another challenge method; however, it was much more delayed in its expression (Cutler et al., 2001). In the study of this report, horses were examined in the same manner at all examination times, including the same handlers, protocol, and examination area. Hence, it is unclear whether this observation reflects a true characteristic of the disease model, or some other unidentified event.

Interpretation of neurologic abnormalities of young horses can be challenging. However, the examiners of this study have considerable experience in this field, and they considered the clinical neurologic abnormalities observed to be unequivocal. The high degree of agreement between the examiners as well as the observation that clinical abnormalities persisted and were observed in the same horses at each examination period after being noted, suggests that misclassification of individuals was unlikely.

Seroconversion in control animals was detected at day 14 in the present study, which is similar to that reported previously (Sofaly et al., 2002). Treatment with ponazuril at 5.0 mg/kg reduced the proportion of horses demonstrating seroconversion from 100% in the control group to 40%. We presume that the ponazuril given the treated horses killed the parasite before its migration from the gastrointestinal tract into somatic tissues, where antigen presentation could occur. Furthermore, ponazuril has been demonstrated at a dose of 5.0 mg/kg to achieve steady-state blood concentrations of 4.3 mg/L after 7 days of treatment (Furr and Kennedy, 2001), a concentration that is adequate to kill *S. neurona* in vitro (Lindsay et al., 2000). Hence, any ex-

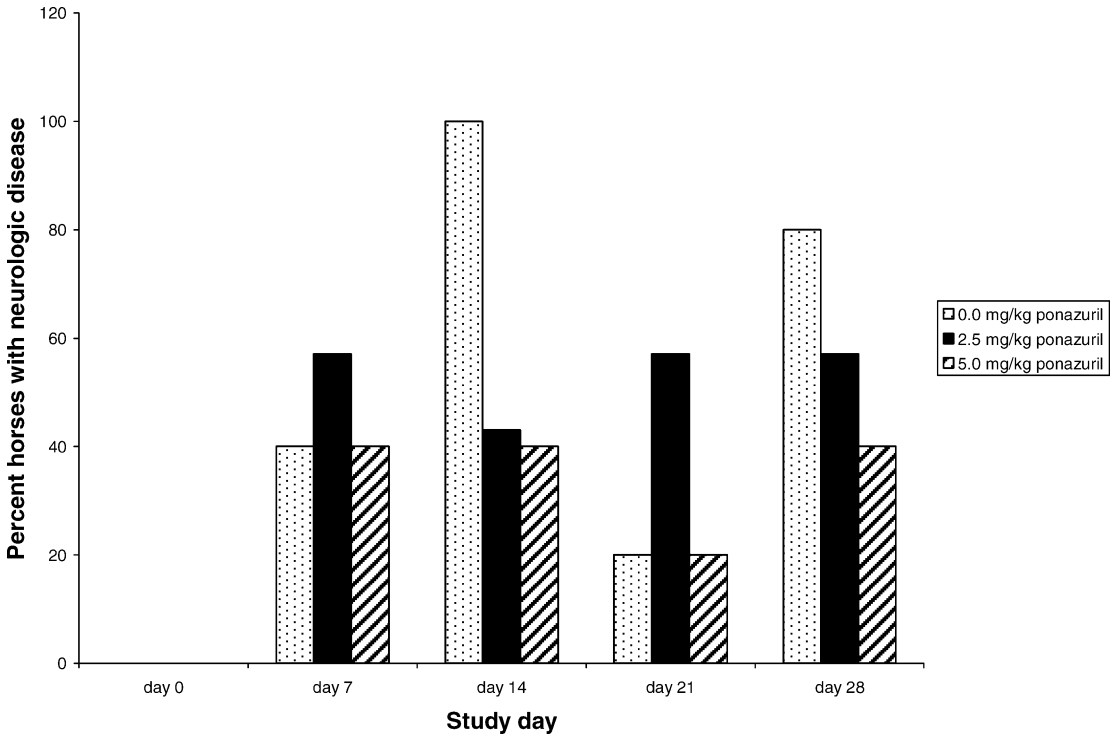

FIGURE 1. Percentage of horses that were treated with ponazuril and challenged with *S. neurona*, which demonstrated neurologic abnormalities. Over all days, the incidence of clinical illness was statistically different among groups at $P = 0.034$.

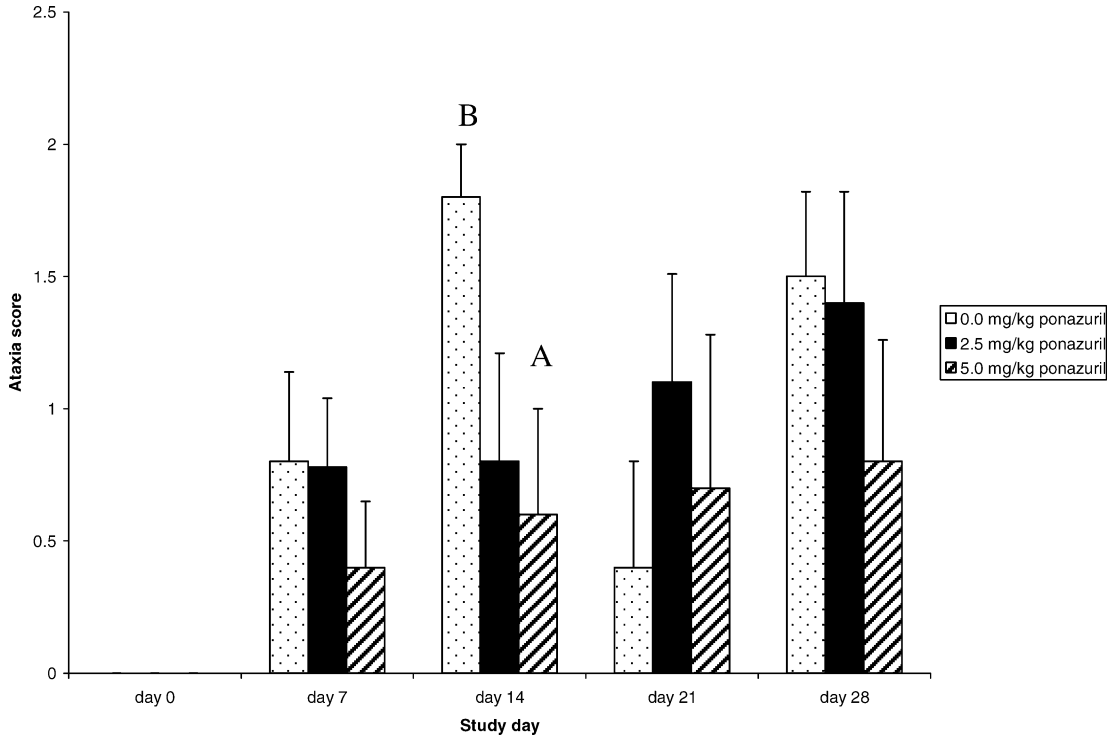

FIGURE 2. Ataxia severity score for horses treated with various doses of ponazuril and challenged with *S. neurona*. Bars with different letters are statistically different at $P < 0.05$.

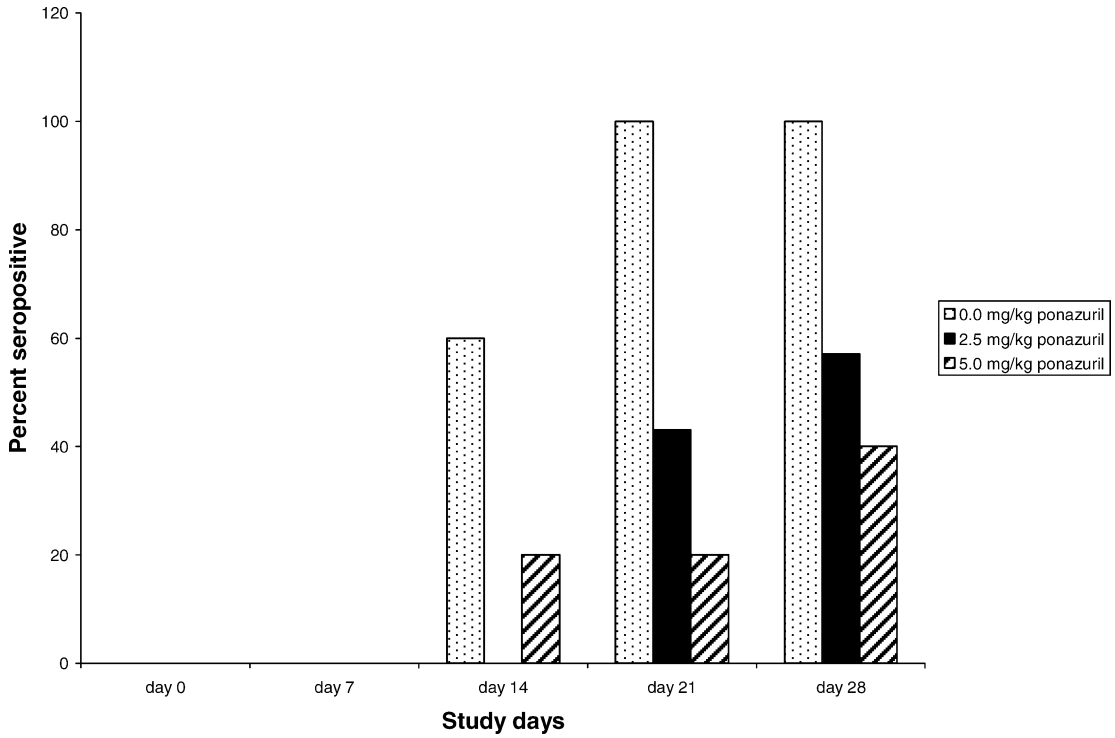

FIGURE 3. Percentage of animals, by group and study day that were seropositive after challenge with *S. neurona* and treated with various dosages of ponazuril. Over all study days, the incidence of seroconversion did differ among treatment groups at a $P = 0.028$.

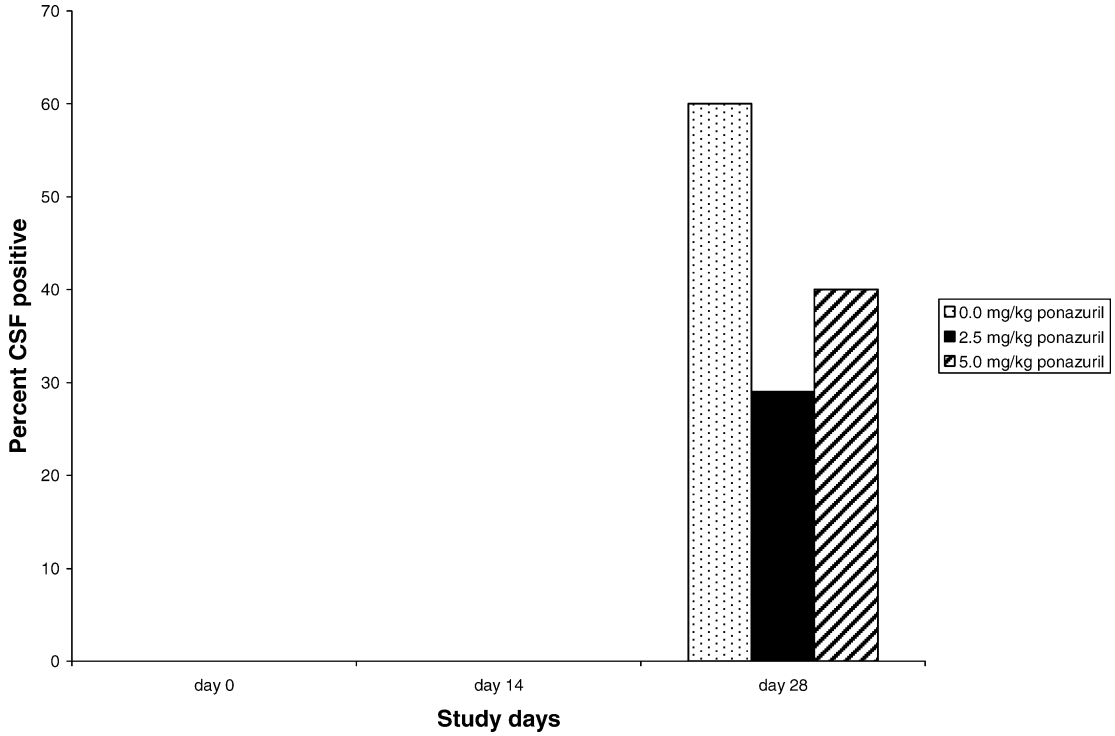

FIGURE 4. Percentage of horses, by group and study day, that were treated with various dosages of ponazuril and challenged with *S. neurona*, which were positive in CSF by WB. Incidence of WB positive did not differ among treatment groups ($P > 0.05$).

TABLE I. Red blood cell counts, nucleated cell counts, and total protein in the CSF of horses treated with various doses of ponazuril and challenged with *S. neurona.* Numbers given are mean, standard deviation (parentheses), and range.

	Study day								
	0			14			28		
Ponazuril (mg/kg)	0.0	2.5	5.0	0.0	2.5	5.0	0.0	2.5	5.0
n	5	7	5	5	7	5	5	7	5
RBC	1.3	0.3	1.0	15.2	7.6	4.6	9.5	3.4	3.0
(cells/μl)	(0.9)	(0.21)	(0.5)	(12.0)	(4.8)	(1.6)	(6.9)	(1.1)	(1.0)
	0–140	0-85	0-3	0-63	0–36	0–8	0–137	0–5	1–6
Nucleated	2.8	1.1	0.2	0.6	0.1	0	0.4	2.1	0
(cells/μl)	(2.3)	(0.5)	(0.2)	(0.4)	(0.1)	(0)	(0.2)	(1.2)	(0)
	0–12	0–4	0–1	0–2	0–1	0–0	0–1	0–9	0–1
Total protein	59.2	53.0	53.8	52.6	56.3	64.4	57.4	57.4	57.0
(mg/dl)	(3.0)	(4.0)	(6.1)	(1.4)	(5.1)	(8.5)	(1.4)	(4.5)	(4.4)

traintestinal *S. neurona* should have been killed before its invasion of the CNS.

Results of CSF evaluation for total protein and cell counts did not detect any differences between treatment groups, nor did individual clinically affected animals demonstrate changes in CSF total protein concentration, or cell counts. This finding confirms previous observations in clinically affected animals that these parameters have little value in the diagnosis of EPM. Failure to demonstrate organisms within the CNS, even when significant disease is present, is a consistent feature of the EPM challenge models (Fenger et al., 1997; Cutler et al., 2001; Saville et al., 2001). It has been proposed that the horse clears the organism from the CNS very quickly; hence, a delayed examination, as conducted here, contributes to the inability to document organisms (Saville et al., 2004). Additional studies are needed to confirm or refute this hypothesis.

A variety of recommendations have been made to minimize the development of EPM in horses. These recommendations include approaches to minimize exposure to the parasite in the horses' environment (Johnson, 1998), vaccination, or prophylactic treatment. Diclazuril, a triazine derivative anticoccidial compound, has been evaluated in IFN-KO mice for its ability to prevent infection with *S neurona* (Dubey, Fritz et al., 2001). Continued daily treatment with diclazuril, beginning before infection, prevented development of neural sarcocystosis, yet efficacy decreased when the drug was given several days after infection (Dubey, Fritz et al., 2001). Reports of the evaluation of diclazuril as a preventive in horses with EPM could not be found. Pyrantel tartrate has been proposed as well, but it confers no protection upon IFN-KO mice given *S. neurona* sporocysts, nor does it reduce the infection rate in horses when fed daily at the conventional dosage (2.6 mg/kg/day) (Lindsay and Dubey, 2001; Rossano et al., 2005). Ponazuril has previously been investigated in IFN-KO mice as a single dose after infection (Franklin et al., 2003). This dose resulted in partial but not complete protection (Franklin et al., 2003).

Ponazuril as used in this study did not completely arrest infection or clinical illness resulting from infection. Reasons for this finding were not examined in this study. However, the challenge dose in these horses was high (1 million sporocysts), a number that likely far exceeds any one-time natural exposure. In addition, these horses were immunocompromised by lengthy transport, increasing susceptibility to infectious disease. Hence, it is likely that the challenge to these horses was greater than that which horses in most situations are likely to experience. Given the observation in experimental challenge models that fewer organisms are associated with less severe disease (Sofaly et al., 2002), it is possible that ponazuril may perform better in the field than demonstrated in this study. The optimal use of ponazuril in field situations is not identified, but a course of treatment before or concordant with major stressful events would seem prudent. Duration of treatment necessary for effective prophylaxis also is not identified in this study. The effectiveness of ponazuril in horses that are already infected (seropositive) has not been established; however, because treatment with 5.0 mg/kg is effective for horses with clinical illness, it would seem likely that it also would have protective effects in these horses.

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